

# Inactivation of the Enzyme GSK3 $\alpha$ by the Kinase IKKi Promotes AKT-mTOR Signaling Pathway that Mediates Interleukin-1-Induced Th17 Cell Maintenance

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## SUMMARY

Interleukin-1 (IL-1)-induced activation of the mTOR kinase pathway has major influences on Th17 cell survival, proliferation, and effector function. Via biochemical and genetic approaches, the kinases IKKi and GSK3 $\alpha$  were identified as the critical intermediate signaling components for IL-1-induced AKT activation, which in turn activated mTOR. Although insulin-induced AKT activation is known to phosphorylate and inactivate GSK3 $\alpha$  and GSK3 $\beta$ , we found that GSK3 $\alpha$  but not GSK3 $\beta$  formed a constitutive complex to phosphorylate and suppress AKT activation, showing that a reverse action from GSK to AKT can take place. Upon IL-1 stimulation, IKKi was activated to mediate GSK3 $\alpha$  phosphorylation at S21, thereby inactivating GSK3 $\alpha$  to promote IL-1-induced AKT-mTOR activation. Thus, IKKi has a critical role in Th17 cell maintenance and/or proliferation through the GSK-AKT-mTOR pathway, implicating the potential of IKKi as a therapeutic target.

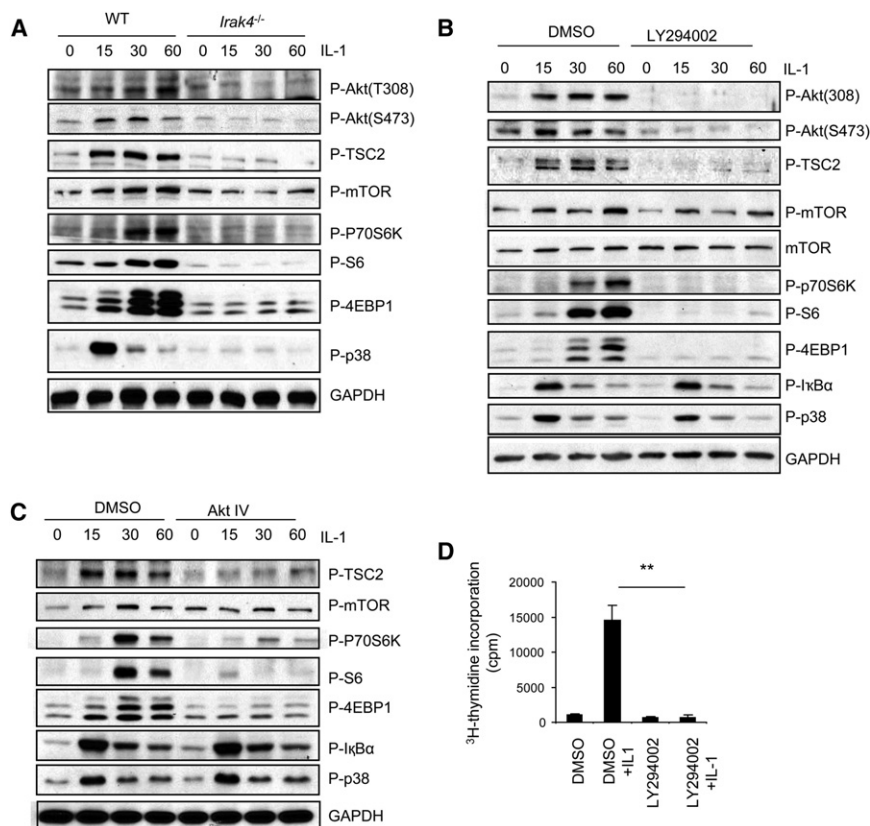
## INTRODUCTION

Invading pathogens are detected by the innate immune system through the recognition of pathogen-associated molecular patterns by various pattern recognition receptors including Toll-like receptors. The activation of pattern recognition receptors induces the production of inflammatory cytokines such as IL-1 and TNF, leading to inflammatory responses (Kawai and Akira, 2007). Activation of professional antigen-presenting

cells through the pattern recognition receptors leads to the onset of adaptive immunity, triggering the differentiation and activation of CD4<sup>+</sup> T helper (Th) lymphocytes, which is essential in regulating immune responses and autoimmune and inflammatory diseases (Janeway and Medzhitov, 2002). Th17 cells have been defined as a distinct lineage of CD4<sup>+</sup> Th cells that produce IL-17, IL-17F, IL-21, and IL-22 (Langrish et al., 2005; Bettelli et al., 2006; Harrington et al., 2005; Mangan et al., 2006; Park et al., 2005; Veldhoen et al., 2006). Th17 cells have attracted tremendous attention in immunology as a result of their important function in host defense against bacterial and fungal infection and potent pathogenic role in autoimmune and inflammatory diseases (Langrish et al., 2005; Park et al., 2005).

Several cytokines including TGF- $\beta$  and IL-6 are required for Th17 cell differentiation upon T cell receptor (TCR) activation. Transcription factors including STAT3 together with lineage-specific factors ROR $\alpha$  and ROR $\gamma$ t direct Th17 cell development and effector function through induction of a set of signature cytokines and cytokine receptors, including IL-23R and IL-1R (Korn et al., 2009; Wei et al., 2007; Ivanov et al., 2006; McGeachy et al., 2007). Although the detailed molecular mechanism is still unclear, mice lacking either IL-1 or IL-23 are resistant to disease induction in Th17 cell-dependent collagen-induced arthritis (CIA), experimental autoimmune encephalomyelitis (EAE), and inflammatory bowel disease (IBD) (Ben-Sasson et al., 2009; Sutton et al., 2006; Murphy et al., 2003; Langrish et al., 2005; Chung et al., 2009).

Although the detailed molecular mechanism is still unclear, IL-1 has major influences on Th17 cell development and effector function, possibly through cell proliferation and survival to maintain the differentiated state of Th17 cells (Korn et al., 2009; Gulen et al., 2010). IL-1 stimulation leads to strong and prolonged activation of the mammalian target of rapamycin



**Figure 1. PI3K and AKT Are Required for IL-1-Induced mTOR Activation**

(A) Cell lysates from wild-type and *Irak4*<sup>-/-</sup> Th17 cells untreated or treated with IL-1 (10 ng/ml) for different time points were analyzed by protein blot analysis with antibodies as indicated. (B and C) Wild-type Th17 cells were untreated or treated with IL-1 (10 ng/ml) for different time points in the presence and absence of 10  $\mu$ M PI3K inhibitor (LY294002) (B) and 1  $\mu$ M AKT inhibitor (AKT IV) (C). Cell lysates were analyzed by protein blot analysis with antibodies as indicated. (D) Wild-type Th17 cells were rested overnight, followed by incubation with 10 ng/ml IL-1 in the presence and absence of 10  $\mu$ M PI3K inhibitor (LY294002). The treated cells were incubated 1 additional day with <sup>3</sup>H for thymidine incorporation experiment. Error bars, SD; \*\*p < 0.01 (two-tailed t test). Data are representative of three independent experiments.

(mTOR) pathway in Th17 cells, including phosphorylation of mTOR and downstream components 4E-BP1 and S6, which play a key role in controlling protein translation. 4E-BP1 is a repressor for protein translation through its interaction with translation initiation factor eIF4E. Phosphorylation of 4E-BP1 by mTOR disrupts its interaction with eIF4E, thereby promoting protein translation, especially on mRNAs with structured 5' UTR including cMyc and Cyclin D1 (Hashemolhosseini et al., 1998; Nelsen et al., 2003). Although mTOR signaling is known to have a major impact on cell growth and proliferation, the mTOR inhibitor rapamycin abolishes IL-1-induced cell proliferation in Th17 cells, indicating the importance of IL-1-induced mTOR activation in IL-1-dependent Th17 cell maintenance and/or proliferation.

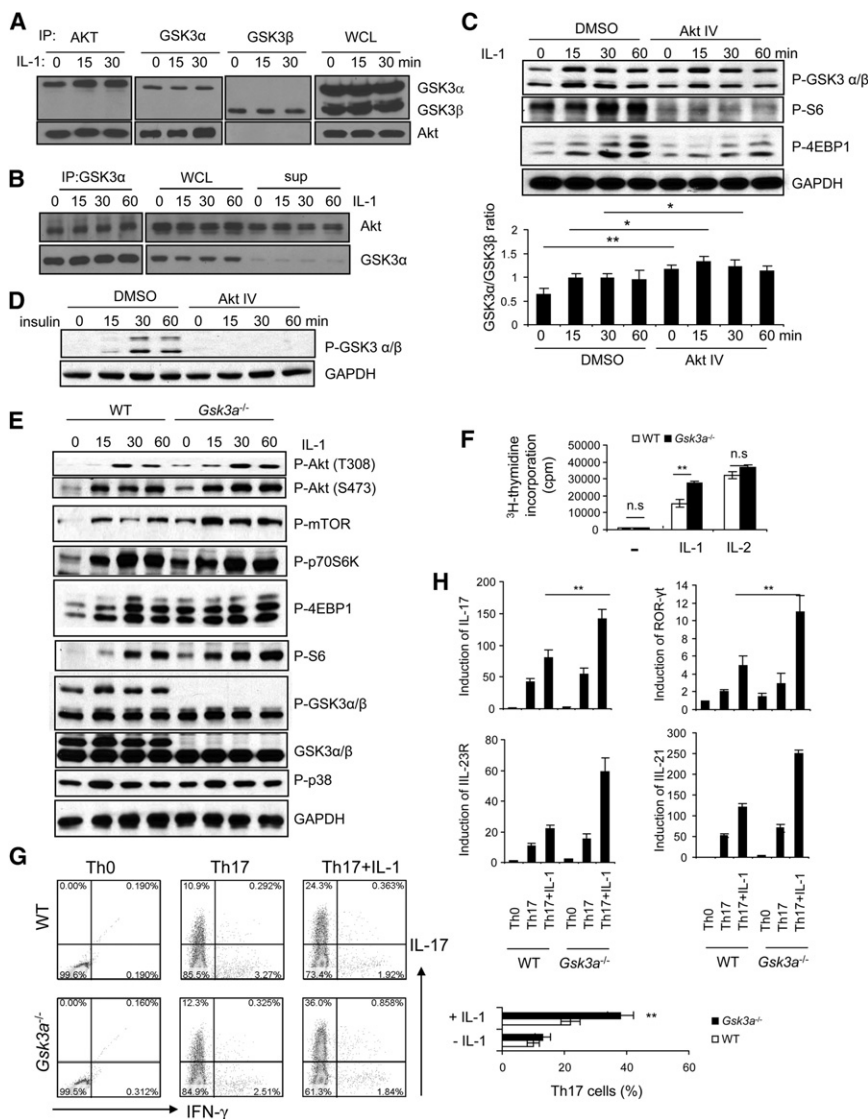
We investigated the intermediate signaling events for IL-1-induced mTOR activation and how this pathway is modulated. We found that AKT was sequestered in a complex with IKKi and glycogen synthase kinase 3 $\alpha$  (GSK3 $\alpha$ ) before IL-1 stimulation. GSK3 $\alpha$  negatively regulates AKT activation by phosphorylating AKT at T312 in the substrate binding site, which inhibited IL-1-induced AKT activation and function. Importantly, although IKKi constitutively interacted with GSK3 $\alpha$ , IL-1 stimulation induced the recruitment of IKKi to the TRAF6-TAK1 signaling complex. IKKi (also known as IKK $\epsilon$  or I kappaB kinase  $\epsilon$ ) is then activated to mediate IL-1-induced GSK3 $\alpha$  phosphorylation at S21 and consequent inactivation of GSK3 $\alpha$ , resulting in AKT-mTOR activation. It is important to note that whereas PI3K and AKT are known to

be upstream kinases for insulin-induced GSK3 phosphorylation and inactivation, this study provides an example that a reverse action from GSK to AKT can take place. We also demonstrate a distinct role of GSK3 $\alpha$  versus GSK3 $\beta$  in IL-1 signaling, implicating the specific function of GSK3 $\alpha$  in autoimmune inflammatory responses. The IL-1-induced IKKi-GSK3 $\alpha$ -mediated AKT-mTOR axis is probably not only critical for the modulation of Th17 cell proliferation and Th17 cell-dependent autoimmunity, but also is important for coordinated regulation of immune responses and cell metabolism.

## RESULTS

### PI3K and AKT Are Required for IL-1-Induced mTOR-Dependent Th17 Cell Proliferation

PI3K and AKT are upstream positive regulators of mTOR through the phosphorylation of TSC2 and consequent disruption of TSC1/TSC2 heterodimer (Manning et al., 2002; Inoki et al., 2002). IL-1 stimulation indeed induced AKT (T308 and S473) and TSC2 (T1462) phosphorylation in wild-type Th17 cells, which was abolished in IRAK4-deficient Th17 cells (Figure 1A). To test the necessity of PI3K activity in IL-1-induced mTOR activation, Th17 cells were stimulated with IL-1 in the presence or absence of PI3K (LY294002) and AKT (AKTIV) inhibitors. LY294002 and AKTIV effectively inhibited IL-1-induced phosphorylation of mTOR and downstream components p70S6K, S6, and 4EBP1 (Figures 1B and 1C), whereas phosphorylation of the kinases I $\kappa$ B $\alpha$  and p38 was not affected. Furthermore, IL-1-induced survival and/or proliferation of Th17 cells was also substantially reduced in the presence of LY294002 (Figure 1D), indicating the requirement of the PI3K-AKT axis in IL-1-mediated mTOR activation and Th17 cell maintenance and/or proliferation.



### IL-1-Induced GSK3 $\alpha$ Phosphorylation Is Independent of PI3K-AKT Kinase Activity

To identify intermediate signaling components that mediate IL-1-induced AKT activation, we searched for AKT-interacting proteins. Coimmunoprecipitation of lysates from 293 cells transfected with IL-1R (293-IL-1R) showed that AKT interacted with GSK3 $\alpha$ , but not GSK3 $\beta$  (Figure 2A). IL-1 stimulation further induced the interaction of AKT with GSK3 $\alpha$ . GSK3 $\alpha$  and GSK3 $\beta$  were not coimmunoprecipitated, suggesting that they are probably not in the same complex (Figure 2A). Furthermore, when GSK3 $\alpha$  was depleted in the supernatant after two cycles of immunoprecipitation, there was still a substantial amount of AKT in the supernatant, implying that GSK3 $\alpha$ -AKT represents one of the AKT complexes in the cells (Figure 2B). The activated AKT upon insulin stimulation are known to phosphorylate GSK3 $\alpha$  (S21) and GSK3 $\beta$  (S9), inactivating GSK3 activity, which then removes its inhibitory effect on glycogen synthase (Cross et al., 1995). IL-1 stimulation also induced phosphorylation of GSK3 $\alpha$  (S21) and GSK3 $\beta$  (S9) in wild-type Th17 cells, which

### Figure 2. IL-1R-Mediated GSK3 $\alpha$ Phosphorylation Is Independent on PI3K-AKT Kinase Activity

(A) Lysates from 293-IL-1R cells untreated or treated for 15 or 30 min with IL-1 (1 ng/ml) were immunoprecipitated with anti-AKT, anti-GSK3 $\alpha$ , and anti-GSK3 $\beta$ , followed by protein blot analysis. WCL, whole cell lysates.

(B) GSK3 $\alpha$  was pulled down with two rounds of immunoprecipitation. Immunoprecipitated complex, whole cell lysate, and the remaining supernatant were analyzed by protein blot analysis with GSK3 $\alpha$  and AKT1 antibody.

(C) Wild-type Th17 cells were untreated or treated with IL-1 (10 ng/ml) for different time points in the presence and absence of 1  $\mu$ M AKT inhibitor (AKT IV). Cell lysates were analyzed by protein blot analysis with antibodies as indicated. Densitometry quantitation of pooled data is plotted below.

(D) Wild-type bone marrow macrophages were untreated or treated with insulin (10 nM) for different time points in the presence and absence of 1  $\mu$ M AKT inhibitor (AKT IV). Cell lysates were analyzed by protein blot analysis with antibodies as indicated.

(E) Cell lysates from wild-type and Gsk3 $\alpha^{-/-}$  Th17 cells untreated or treated with IL-1 (10 ng/ml) for different time points were analyzed by protein blot analysis with antibodies as indicated.

(F) Wild-type and Gsk3 $\alpha^{-/-}$  Th17 cells were rested overnight, followed by incubation with 10 ng/ml IL-1 or IL-2 for 3 additional days and with  $^3$ H for 1 more day for thymidine incorporation experiment.

(G) Naive wild-type, Gsk3 $\alpha^{-/-}$  CD4 $^{+}$  T cells (CD4 $^{+}$ CD44 $^{lo}$ ) were polarized to Th17 cells in the presence and absence of IL-1 $\beta$ , followed by intracellular cytokine staining for IL-17 and IFN- $\gamma$ .

(H) Real-time PCR analysis of relative expression of IL-17, IL-21, IL-23R, and ROR $\gamma$ t in wild-type and Gsk3 $\alpha^{-/-}$  Th17 cells as compared to the Th0 cells.

Error bars in (F)–(H) indicate SD; \*\*p < 0.01 (two-tailed t test). All data are representative of three independent experiments.

was abolished in IRAK4-deficient cells (Figure S1 available online). Whereas PI3K and AKT inhibitors completely blocked insulin-induced GSK3 $\alpha$  and GSK3 $\beta$  phosphorylation, both inhibitors partially attenuated IL-1-induced GSK3 $\beta$  but not GSK3 $\alpha$  phosphorylation (Figures 2C, 2D, and S2A). The inhibition of AKTIV on IL-1-induced GSK3 $\beta$  phosphorylation is consistent with the previous finding that AKT mediates GSK3 $\beta$  phosphorylation to remove the inhibitory effect of GSK3 $\beta$  on mTOR activation (Cross et al., 1995). In contrast, these results indicate differential roles of GSK3 $\alpha$  and GSK3 $\beta$  in IL-1 signaling.

### GSK3 $\alpha$ Negatively Modulates IL-1-Induced AKT-mTOR Activity

An important question was then whether and how GSK3 $\alpha$  contributes to IL-1-dependent effector function in Th17 cells. We first examined the impact of GSK3 $\alpha$  deficiency on IL-1-mediated signaling in Th17 cells. Whereas p38 phosphorylation was not affected, both constitutive and IL-1-induced AKT-mTOR activation (phosphorylation of AKT, mTOR, p70S6K,



and S6) were substantially enhanced in GSK3 $\alpha$ -deficient Th17 cells compared to that in wild-type cells (Figures 2E and S2B). Consistent with the increased mTOR activity, GSK3 $\alpha$  deficiency resulted in enhanced IL-1-mediated Th17 cell maintenance and/or proliferation (Figure 2F). GSK3 $\alpha$  did not have much impact on the response of Th17 cells to IL-2, indicating the specific function of this component in the IL-1 pathway (Figure 2F). WT and *Gsk3a*<sup>-/-</sup> naive T cells were differentiated into Th17 cells with or without IL-1 $\beta$  stimulation. Similar numbers of IL-17-positive cells (10%–12%) were detected in WT and *Gsk3a*<sup>-/-</sup> T cells under TGF- $\beta$ +IL-6-mediated differentiation condition (in the absence of IL-1 $\beta$  stimulation) (Figure 2G). Importantly, although IL-1 $\beta$  promoted Th17 cell differentiation in both WT and *Gsk3a*<sup>-/-</sup> T cells, addition of IL-1 $\beta$  resulted in a significantly higher IL-17-positive population in *Gsk3a*<sup>-/-</sup> T cells than that in WT T cells (Figure 2G). Consistent with flow cytometry analysis, the expression of other Th17 cell-associated molecules (including IL-17, ROR $\gamma$ t IL-21, and IL-23R) was also much higher in *Gsk3a*<sup>-/-</sup> T cells than that in WT cells in the presence of IL-1 $\beta$  stimulation (Figure 2H). Taken together, these results suggest that GSK3 $\alpha$  actually negatively regulates IL-1-induced AKT-mTOR activity and consequently suppresses IL-1-dependent Th17 cell differentiation and survival/proliferation. The fact that IL-1 induces specific interaction of GSK3 $\alpha$  with PI3K and AKT (Figure 2A) suggests that GSK3 $\alpha$  might act upstream of PI3K and AKT and modulate their activity in response to IL-1 stimulation, which is consistent with the observation that GSK3 $\alpha$  phosphorylation was not inhibited by PI3K and AKT inhibitors (Figure 2C). Importantly, whereas IL-1 stimulation led to increased AKT and S6 phosphorylation in *Gsk3a*<sup>-/-</sup> MEFs, the insulin-induced AKT and S6 phosphorylation was comparable in wild-type and *Gsk3a*<sup>-/-</sup> MEFs (Figure S2C). These results support the notion that GSK3 $\alpha$  has an inhibitory role in IL-1-induced but not insulin-induced AKT activation.

### GSK3 $\alpha$ Phosphorylates AKT and Inhibits Its Kinase Activity

We then explored how GSK3 $\alpha$  inhibits IL-1-induced AKT-mTOR activation. Through coimmunoprecipitation of lysates from 293-IL-1R cells with anti-GSK3 $\alpha$  or anti-GSK3 $\beta$  followed by in vitro kinase assay, it was noted that GSK3 $\alpha$ , but not GSK3 $\beta$ , immunoprecipitates phosphorylated a protein at the size of AKT (Figure 3A). These results are consistent with the observation that AKT was coimmunoprecipitated only with GSK3 $\alpha$ , not with GSK3 $\beta$  (Figure 2A). In vitro kinase assay with recombinant proteins showed that GSK3 $\alpha$  indeed promoted AKT phosphorylation (Figure 3B). Tandem MS revealed that GSK3 $\alpha$  promoted AKT phosphorylation at T312 (Figure 3C). Figure S3 showed the structure of active AKT kinase domain (green) bound to the substrate GSK3 $\beta$  peptide (cyan). Phosphorylation of the activation loop at T308 (blue with phosphate group in red) is essential for AKT activation. T312 (yellow) of AKT is in the substrate binding site and the phosphorylation on its side chain hydroxyl group (red) will cause steric clash with the substrate, thereby inhibiting the AKT kinase activity. To assess the impact of phosphorylation of T312 on AKT activation and function, we mutated T312 to aspartic acid (D) to mimic phosphorylation and transfected empty vector, flag-tagged wild-type, and AKT mutant (AKT<sup>T312D</sup>) into 293-IL-1R cells. The cell lysates from

the transfected cells were immunoprecipitated with anti-flag (M2) to pull down wild-type and mutant AKT, followed by in vitro kinase assay with recombinant GSK3 $\beta$  as a substrate. As shown in Figure 3D, AKT<sup>T312D</sup> displayed attenuated kinase activity compared to wild-type AKT, demonstrating that GSK3 $\alpha$ -mediated phosphorylation of T312 is inhibitory for AKT activation and function.

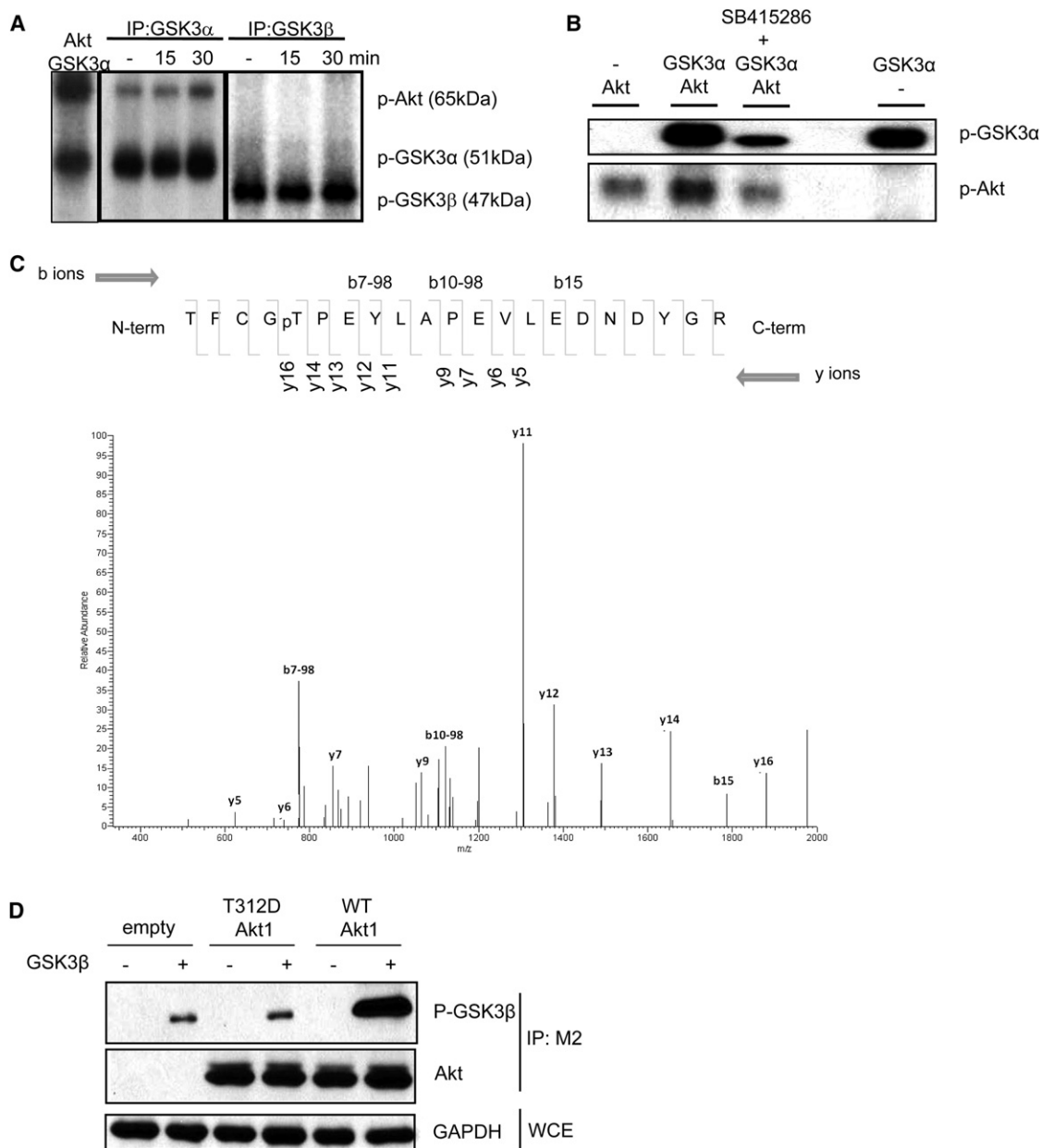
### IL-1 Induces IKKi Activation to Mediate GSK3 $\alpha$ Phosphorylation

Because IL-1-induced GSK3 $\alpha$  phosphorylation was not inhibited by PI3K and AKT inhibitors, a different kinase probably functions upstream of GSK3 $\alpha$  and is responsible for the phosphorylation of GSK3 $\alpha$  in response to IL-1 stimulation. Through coimmunoprecipitation of lysates from 293-IL-1R cells, we found that GSK3 $\alpha$ , but not GSK3 $\beta$ , formed a complex with IKKi (Figure 4A). Importantly, whereas I $\kappa$ B $\alpha$  phosphorylation was intact, IL-1-induced GSK3 $\alpha$  and GSK3 $\beta$  phosphorylation was greatly reduced in IKKi-deficient Th17 cells compared to that in wild-type cells (Figure 4B), implying that IKKi might be the upstream kinase of GSK3 in response to IL-1 stimulation.

Upon IL-1 stimulation, adaptor MyD88 is recruited to the IL-1 receptor, followed by the recruitment of serine and threonine kinases IRAKs (interleukin-1 receptor-associated kinases) and TRAF6. IRAK4 mediates the phosphorylation of IRAK1 and IRAK2, followed by the activation of TAK1 and IKK in a TRAF6-dependent manner, leading to NF- $\kappa$ B activation (Yao et al., 2007). Interestingly, we found that the IL-1 stimulation induced the interaction of TRAF6-TAK1 with the IKKi-GSK3 $\alpha$  complex in 293-IL-1R cells (Figure 4C). Because IKKi is important in IL-1-induced GSK3 phosphorylation, we examined IL-1-mediated IKKi activation. We immunoprecipitated IKKi from cell lysates of MEFs and primary kidney epithelial cells untreated and treated with IL-1, followed by in vitro kinase assay. Increased IKKi phosphorylation was detected in lysates from both cell types 10–15 min after IL-1 stimulation (Figure 4D), demonstrating IL-1-induced IKKi activation in cell culture model. IL-1-induced IKKi autophosphorylation was substantially reduced in IRAK4-deficient kidney epithelial cells and TRAF6- and TAK1-deficient MEFs. These results indicate that IL-1 stimulation specifically leads to IKKi activation in an IRAK4-TRAF6-TAK1-dependent manner. We then examined whether IKKi can directly phosphorylate GSK3 in vitro. Both GSK3 $\alpha$  and GSK3 $\beta$  immunoprecipitated from 293-IL-1R cells were subjected to in vitro kinase assay with or without recombinant IKKi protein. Importantly, we observed that recombinant IKKi was able to effectively phosphorylate GSK3 $\alpha$  but not GSK3 $\beta$  in vitro (Figure 4E). Thus, IKKi directly phosphorylates only GSK3 $\alpha$ , whereas the impact of IKKi on IL-1-induced GSK3 $\beta$  phosphorylation is likely to be indirect, probably through the activation of an intermediate kinase(s). In support of this, recombinant IKKi was also able to phosphorylate only recombinant GSK3 $\alpha$  but not GSK3 $\beta$  in an in vitro kinase assay, indicating that IKKi is a direct upstream kinase of GSK3 $\alpha$  (Figure 4F).

### IKKi-Mediated Inactivation of GSK3 $\alpha$ Is Required for IL-1-Induced AKT-mTOR Activation

We next attempted to identify the IKKi-mediated phosphorylation sites of GSK3 $\alpha$  by tandem mass spectrometry. A GSK3 $\alpha$



**Figure 3. GSK3 $\alpha$  Phosphorylates and Inhibits AKT**

(A) Lysates from 293-IL-1R cells untreated or treated for 15 or 30 min with IL-1 (1 ng/ml) were immunoprecipitated with anti-GSK3 $\alpha$  and anti-GSK3 $\beta$ , followed by in vitro kinase reaction in the presence (first lane) or absence of recombinant AKT.

(B) Recombinant AKT and GSK3 $\alpha$  proteins were incubated in kinase reaction in the presence and absence of GSK3 inhibitor (30  $\mu$ M SB415286).

(C) Tandem mass spectrometry (ms2) of precursor ions in the AKT1 phosphopeptide (amino acids 308–328; sequence TFCGpTPEYLAPEVLEDNDYGR).

(D) 293-IL-1R cells were transfected with flag-tagged wild-type AKT1, T312D mutant AKT1, or empty vectors. The cell lysates from the transfected cells were immunoprecipitated with anti-flag antibody (M2), followed in vitro kinase assay with recombinant GSK3 $\beta$  as a substrate.

Data are representative of at least three separate experiments.

peptide of amino acids 19–50 (TSSFAEPGGGGGGGGGGPGG SASGPGGTGGGK) was phosphorylated on S21 (Figure 4G). Previous studies have shown that phosphorylation of S21 results in the N-terminal domain of GSK3 $\alpha$  interacting with its phosphate binding pocket, preventing recognition of primed substrates (Frame et al., 2001). Therefore, it is possible that IKKi-mediated GSK3 $\alpha$  phosphorylation at S21 also attenuates GSK3 $\alpha$  kinase

activity, thereby removing the inhibitory effect of GSK3 $\alpha$  on IL-1-induced AKT-mTOR activity. To test this hypothesis, we employed GSK3 $\alpha$  knockin mice in which S21 was mutated to alanine. Whereas IL-1-induced p38 phosphorylation was intact, AKT (T308 and S473)-mTOR activation was substantially decreased in *Gsk3a*<sup>21A/21A</sup> Th17 cells as compared to that in wild-type cells (Figure 5A). Consistent with the decreased

mTOR activity, IL-1-mediated cell survival and/or proliferation was reduced in *Gsk3a*<sup>21A/21A</sup> Th17 cells as compared to that in wild-type cells (Figure 5B). WT and *Gsk3a*<sup>21A/21A</sup> naive T cells were differentiated into Th17 cells with or without IL-1 $\beta$  stimulation. Similar numbers of IL-17-positive cells were detected in WT and *Gsk3a*<sup>21A/21A</sup> T cells under TGF- $\beta$ +IL-6-mediated differentiation condition (in the absence of IL-1 $\beta$  stimulation) (Figure 5C). Importantly, although IL-1 $\beta$  promoted Th17 cell differentiation in both WT and *Gsk3a*<sup>21A/21A</sup> T cells, addition of IL-1 $\beta$  resulted in a significantly reduced IL-17-positive population in *Gsk3a*<sup>21A/21A</sup> T cells than that in WT T cells (Figure 5C). Furthermore, the expression of Th17 cell-associated molecules (including ROR $\gamma$ t, IL-17, IL-21, and IL-23R) was also much lower in *Gsk3a*<sup>21A/21A</sup> T cells than that in WT cells in the presence of IL-1 $\beta$  stimulation (Figure 5D). Thus, IKKi-mediated GSK3 $\alpha$  phosphorylation at S21 is indeed required for the inactivation of GSK3 $\alpha$  to remove GSK3 $\alpha$ 's inhibitory effect on AKT, which is critical for IL-1-induced activation AKT-mTOR and Th17 cell maintenance and/or proliferation.

#### IKKi Deficiency Leads to Loss of IL-1-Induced AKT-mTOR Activation

We examined the direct impact of IKKi deficiency on IL-1-mediated AKT-mTOR pathway and IL-1-dependent Th17 cell maintenance and/or proliferation. IL-1-induced phosphorylation of AKT (T308), TSC2, mTOR, and downstream components p70S6K, S6, and 4EBP1 were markedly reduced in IKKi-deficient Th17 cells compared to that in wild-type cells (Figure 6A). Consistent with the decreased AKT-mTOR activation, IKKi deficiency substantially reduced IL-1-mediated impact on Th17 cell maintenance and/or proliferation compared to that in wild-type cells (Figure 6B). However, IKKi deficiency did not have much impact on the response of Th17 cells to IL-2, indicating the specific function of IKKi in IL-1 pathway (Figure 6B). Although IKKi deficiency did not have much impact on TGF- $\beta$ +IL-6-mediated Th17 cell differentiation, addition of IL-1 $\beta$  resulted in a markedly reduced IL-17-positive population in *Ikkbke*<sup>-/-</sup> T cells than that in WT T cells (Figure 6C). Furthermore, the expression of Th17 cell-associated molecules (including ROR $\gamma$ t, IL-17, IL-21, and IL-23R) was also much lower in *Ikkbke*<sup>-/-</sup> T cells than that in WT cells in the presence of IL-1 $\beta$  stimulation (Figure 6D). These results suggest that IKKi not only functions as an upstream kinase of GSK3 $\alpha$  but also plays a critical role in IL-1-induced AKT-mTOR activation in Th17 cells and consequent Th17 cell maintenance and/or proliferation (Figure S5).

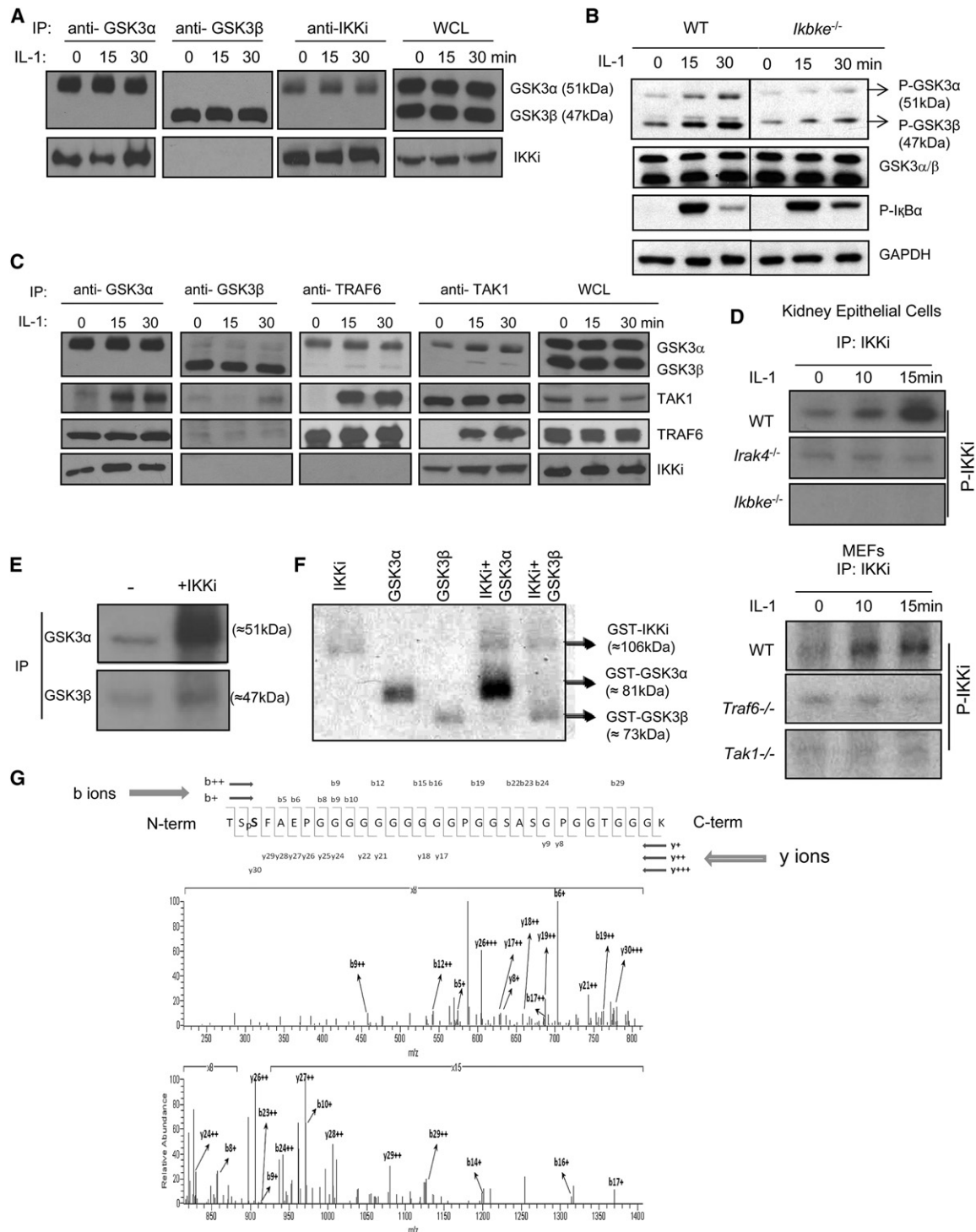
An important question is how is AKT activated upon IL-1 stimulation. IKKi can directly mediate AKT phosphorylation and activation in response to EGF stimulation (Xie et al., 2011; Guo et al., 2011). Because IKKi is required for IL-1-induced activation of AKT-mTOR signaling pathway, we examined a possible direct role of IKKi in IL-1-mediated AKT activation. Through coimmunoprecipitation, we found that IKKi forms a complex with AKT (data not shown). Furthermore, we observed that recombinant IKKi was able to effectively phosphorylate AKT in vitro (Figure 6E), indicating that IKKi probably is able to directly phosphorylate AKT.

Because PI3K signaling is required for IL-1-induced AKT-mTOR activation (Figures 1B and 1D), we wondered whether PDK1 might also be involved in IL-1-induced AKT phosphorylation and activation.

We found that IL-1-induced PDK1 phosphorylation was detected in both wild-type and IKKi-deficient Th17 cells, indicating that PDK1 activation is IKKi independent (Figure 6A). Although IKKi can directly phosphorylate AKT, it is unclear whether PDK1 is also required for IL-1-induced AKT activation. We found that IL-1-induced GSK3, JNK, and I $\kappa$ B $\alpha$  phosphorylations were intact in PDK1-deficient colon cancer cells, whereas IL-1-induced phosphorylation of AKT T308 and downstream components of mTOR (p70S6K, S6, and 4EBP1) were abolished in the absence of PDK1 (Figure 6F). PDK1 deficiency did not have much impact on IL-1-induced AKT phosphorylation at S473 (Figure 6F), which might be directly phosphorylated by IKKi (Xie et al., 2011; Guo et al., 2011).

#### The Impact of IKKi and GSK3 $\alpha$ on Th17 Cell Effector Function in Induction of EAE

IL-1-mediated signaling in T cells is essential for in vivo Th17 cell differentiation and IL-17-dependent autoimmune diseases (Sutton et al., 2006). Here we showed that IKKi functions downstream of IRAK4 to mediate IL-1-induced AKT-mTOR activation and ex vivo Th17 cell survival and/or proliferation. To determine the importance of IKKi on IL-1-mediated impact on Th17 cells in vivo, we transferred MOG-specific Th17 cells from the WT and *Ikkbke*<sup>-/-</sup> mice to sublethally irradiated *Il1r*<sup>-/-</sup> mice, followed by 7 days of injection of either PBS or IL-1 $\beta$ . Whereas IL-1 $\beta$  treatment induced IL-17-producing MOG-specific T cells in *Il1r*<sup>-/-</sup> mice, IKKi deficiency reduced the impact of IL-1 on MOG-specific Th17 cells (Figure 7A). The functionality of MOG-specific Th17 cells from the WT and *Ikkbke*<sup>-/-</sup> mice was also compared by adoptive transfer to WT recipient mice. Compared to MOG<sub>35-55</sub>-specific wild-type Th17 cells, MOG<sub>35-55</sub>-specific IKKi-deficient Th17 cells induced less severe EAE with a delayed onset of disease in recipient mice (Figure 7B). Consistent with the clinical scores, infiltrating CD45<sup>+</sup>, CD4<sup>+</sup>, CD11b<sup>+</sup>, and Gr-1<sup>+</sup> cells were reduced in the brains of mice in which *Ikkbke*<sup>-/-</sup> MOG-specific Th17 cells were transferred compared to those that received wild-type MOG-specific Th17 cells (Figure 7C). Because our results showed that IKKi-mediated AKT-mTOR activation in Th17 cells requires the removal of the inhibitory effector of GSK3 $\alpha$  on AKT, we also examined the effect of GSK3 $\alpha$  deficiency on IL-1-induced Th17 cell maintenance and expansion in vivo and Th17 cell effector function in EAE model. We transferred MOG-specific Th17 cells from WT and CD4Cre*Gsk3a*<sup>fl/fl</sup> mice to sublethally irradiated *Il1r*<sup>-/-</sup> mice, followed by 7 days of injection of either PBS or IL-1 $\beta$ . GSK3 $\alpha$  deficiency enhanced the impact of IL-1 on MOG-specific Th17 cells (Figure 7D). The functionality of MOG-specific Th17 cell from WT and *Gsk3a*<sup>-/-</sup> mice was also compared by adoptive transfer to WT recipient mice to induce EAE. Adoptive transfer of MOG<sub>35-55</sub>-specific *Gsk3a*<sup>-/-</sup> Th17 cells induced more severe EAE in recipient mice compared to WT Th17 cells (Figures 7E and 7F). Thus, GSK3 $\alpha$  deficiency enhanced Th17 effector cell function in vivo, leading to increased severity of EAE. Taken together, these data imply that the IL-1-induced IKKi-GSK3 $\alpha$ -mediated AKT-mTOR axis is critical for the modulation of Th17 effector cell function in vivo, impacting Th17 cell-dependent EAE pathogenesis.



**Figure 4. IL-1 Induces IKKi Activation to Mediate GSK3 $\alpha$  Phosphorylation**

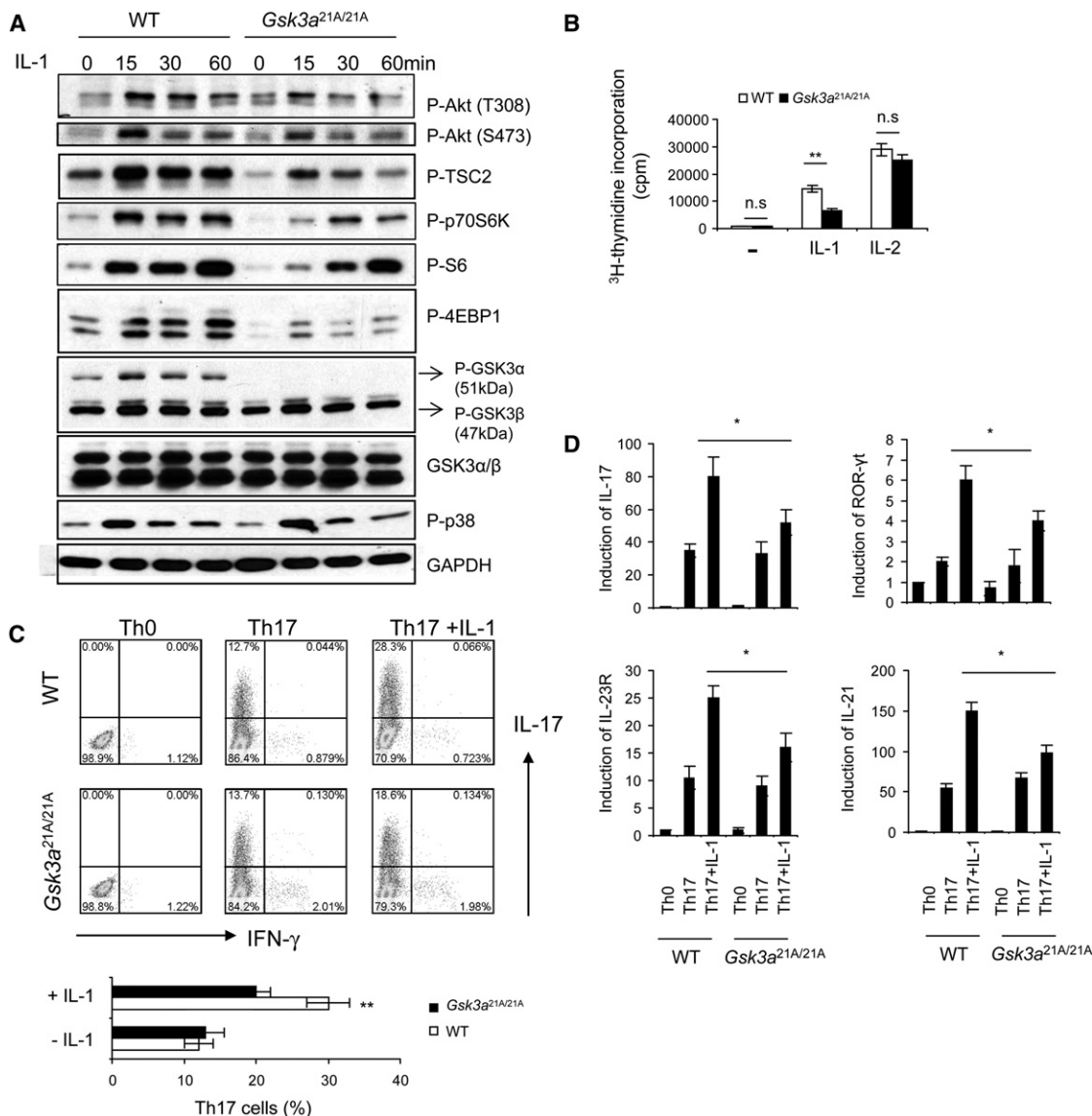
(A) Lysates from 293-IL-1R cells untreated or treated for 15 or 30 min with IL-1 (1 ng/ml) were immunoprecipitated with anti-GSK3 $\alpha$ , anti-GSK3 $\beta$ , and anti-IKKi, followed by protein blot analysis with anti-GSK3 $\alpha$ , anti-GSK3 $\beta$ , anti-IKKi. WCL, whole cell lysates.

(B) Cell lysates from wild-type and *Ikake*<sup>-/-</sup> Th17 cells untreated or treated with IL-1 (10 ng/ml) for different time points were analyzed by protein blot analysis with antibodies as indicated.

(C) Lysates from 293-IL-1R cells untreated (0) or treated for 15 or 30 min with IL-1 (1 ng/ml) were immunoprecipitated with anti-GSK3 $\alpha$ , anti-GSK3 $\beta$ , anti-TRAF6, and anti-TAK1 and analyzed by immunoblot with anti-GSK3 $\alpha$ , anti-GSK3 $\beta$ , anti-TRAF6, and anti-IKKi.

(D) The lysates of kidney epithelial cells from wild-type, *Irak4*<sup>-/-</sup>, and *Ikake*<sup>-/-</sup> mice or cell lysates from wild-type, *Traf6*<sup>-/-</sup>, and *Tak1*<sup>-/-</sup> MEFs were immunoprecipitated with IKKi antibody, followed by in vitro kinase reaction.





**Figure 5. IKKi-Mediated Inactivation of GSK3 $\alpha$  Is Required for IL-1-Induced AKT-mTOR Activation**

(A) Cell lysates from wild-type and *Gsk3a*<sup>21A/21A</sup> Th17 cells untreated or treated with IL-1 (10 ng/ml) for different time points were analyzed by protein blot analysis with antibodies as indicated.

(B) Wild-type and *Gsk3a*<sup>21A/21A</sup> Th17 cells were rested overnight, followed by incubation with 10 ng/ml IL-1 or IL-2 for 3 additional days. The treated cells were incubated 1 additional day with <sup>3</sup>H for thymidine incorporation experiment.

(C) Naive wild-type, *Gsk3a*<sup>21A/21A</sup> CD4<sup>+</sup> T cells were polarized to Th17 cells in the presence and absence of IL-1 $\beta$ , followed by intracellular cytokine staining for IL-17 and IFN- $\gamma$ .

(D) Real-time PCR analysis of relative expression of IL-17, IL-21, IL-23R, and ROR- $\gamma$ t in wild-type and *Gsk3a*<sup>21A/21A</sup> Th17 cells as compared to the Th0 cells.

All data are representative of at least three separate experiments. Error bars in (B) and (D) indicate SD; \*\*p < 0.01 (two-tailed t test).

## DISCUSSION

This study demonstrates a distinct role of GSK3 $\alpha$  versus GSK3 $\beta$  in IL-1 signaling, which has a critical regulatory role in Th17 cell

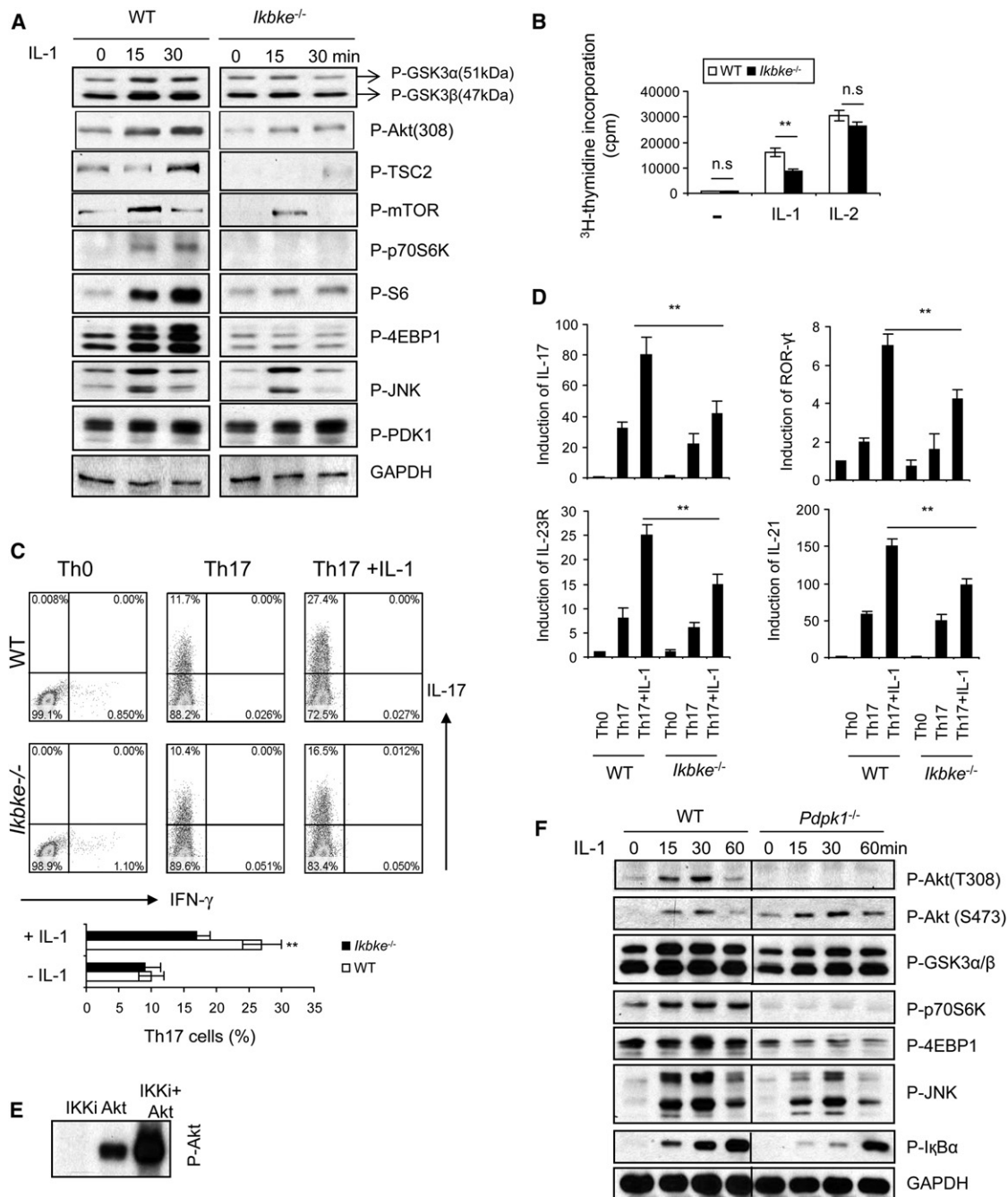
maintenance and/or proliferation. It is important to note that whereas PI3K and AKT are known to be upstream kinases for insulin-induced GSK3 phosphorylation and inactivation, we found here that GSK3 $\alpha$  forms a constitutive complex to suppress

(E) The cell lysates from 293-IL-1R cells were immunoprecipitated with GSK3 $\alpha$  or GSK3 $\beta$  antibody followed by in vitro kinase reaction in the absence and presence of recombinant IKKi protein.

(F) Recombinant GSK3 $\alpha$  and GSK3 $\beta$  proteins were incubated in kinase reaction in the presence and absence of recombinant IKKi protein.

(G) Tandem mass spectrometry (ms2) of precursor ions in the GSK3 $\alpha$  phosphopeptide (amino acids 19–50; sequence TSpSFAEPGGGGGGGGGGPGG SASGPGGTGGGK). All data are representative of at least three separate experiments.





**Figure 6. IKKi Deficiency Leads to Loss of IL-1-Induced AKT-mTOR Activation**

(A) Cell lysates from wild-type and *Ikbke*<sup>-/-</sup> Th17 cells untreated or treated with IL-1 (10 ng/ml) for different time points were analyzed by protein blots with antibodies as indicated.

(B) Wild-type and *Ikbke*<sup>-/-</sup> Th17 cells were rested overnight, followed by incubation with 10 ng/ml IL-1 or IL-2 for 3 additional days. The treated cells were incubated for 1 additional day with <sup>3</sup>H for thymidine incorporation experiment.

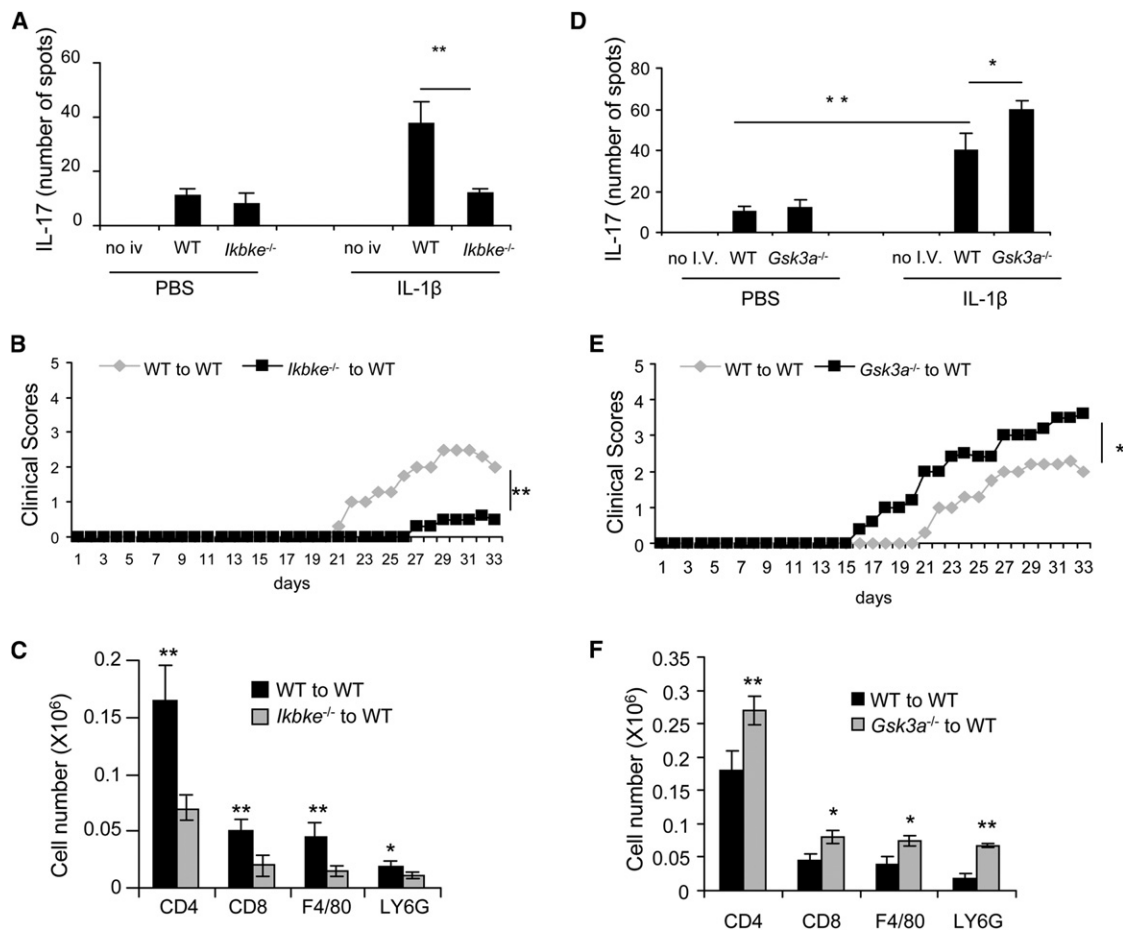
(C) Naive wild-type and *Ikbke*<sup>-/-</sup> CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD44<sup>lo</sup>) were polarized to Th17 cells in the presence and absence of IL-1 $\beta$ , followed by intracellular cytokine staining for IL-17 and IFN- $\gamma$ .

(D) Real-time PCR analysis of relative expression of IL-17, IL-21, IL-23R, and ROR $\gamma$ t in wild-type and *Ikbke*<sup>-/-</sup> Th17 cells as compared to that in Th0 cells.

Data in (A)–(D) are representative of at least three separate experiments. Error bars in (B) and (D) indicate SEM; \*\*p < 0.01 (two-tailed t test).

(E) Recombinant IKKi and AKT1 proteins were incubated in the in vitro kinase reaction.

(F) Cell lysates from wild-type and *Pdpk1*<sup>-/-</sup> DLD1 cells untreated or treated with IL-1 (10 ng/ml) for different time points were analyzed by protein blots with antibodies as indicated.



**Figure 7. IKKi-GSK3 $\alpha$  Axis Is Required for IL-1-Mediated In Vivo Th17 Cell Expansion and Survival**

(A and D) WT, *Gsk3 $\alpha$* <sup>-/-</sup>, and *Ikbke*<sup>-/-</sup> MOG-specific Th17 cells were transferred to *Il1r*<sup>-/-</sup> mice. After 7 days of PBS or IL-1 $\beta$  injection, MOG-specific IL-17-producing cells in the spleen were detected by ELISPOT.

(B and E) Primed MOG<sub>35-55</sub>-specific wild-type, *Gsk3 $\alpha$* <sup>-/-</sup>, and *Ikbke*<sup>-/-</sup> T cells (10 days) were restimulated with MOG<sub>35-55</sub> in vitro in the presence of recombinant IL-23 for 4 days and then transferred to naive wild-type mice. Graph represents the average clinical score after T cell transfer. n = 5; \*\*p < 0.01 (ANOVA).

(C and F) Immune cell infiltration in the brains of wild-type mice transferred with wild-type, *Gsk3 $\alpha$* <sup>-/-</sup>, and *Ikbke*<sup>-/-</sup> Th17 cells was analyzed by flow cytometry (n = 5, 7 days after disease onset).

Error bars (A, C, D, and F) indicate SD; \*p < 0.05; \*\*p < 0.01 (two-tailed t test). All data are representative of three independent experiments.

AKT activation. This study showed that a reverse action from GSK3 $\alpha$  to AKT can take place to impact mTOR activation, whereas previous studies demonstrated how GSK3 $\beta$  can reversely act on mTOR activation by mediating the phosphorylation of TSC2 (Inoki et al., 2006; Wu and Pan, 2010). These pathways probably serve as important links between inflammatory pathways and cell metabolism. Although we have reported the importance of IKKi in IL-17-mediated mRNA stabilization (Bulek et al., 2011), the study here showed that IKKi has a critical role in Th17 cell maintenance and/or proliferation through the GSK-AKT-mTOR pathway, which will have an important impact on the rationale and feasibility for IKKi to be a therapeutic target.

It is well known that insulin receptor signaling results in the activation of PI3K pathway and subsequent phosphorylation of GSK3 $\alpha$  (S21) and GSK3 $\beta$  (S9) by AKT, leading to inactivation of GSK3 activity and removal of its inhibitory effect on glycogen synthase (Cross et al., 1994, 1995, 1997). Therefore, it has been generally accepted that GSK3 is a downstream substrate

of AKT. Our results unexpectedly showed that both PI3K and AKT inhibitors failed to inhibit IL-1-induced GSK3 $\alpha$  phosphorylation, although they partially inhibited GSK3 $\beta$  phosphorylation. GSK3 $\alpha$  and GSK3 $\beta$ , encoded by different genes (Woodgett, 1990), seem to be functionally redundant in some signaling pathways (Doble et al., 2007), although most of the previous studies have focused on GSK3 $\beta$ . An important finding in this study is the identification of the constitutive interaction of GSK3 $\alpha$  (but not GSK3 $\beta$ ) with AKT, which allows GSK3 $\alpha$  to specifically phosphorylate AKT at T312 to suppress AKT activation and function. Although GSK3 $\alpha$  and GSK3 $\beta$  share nearly identical sequences in their kinase domains, a glycine-rich extension at the N terminus of GSK3 $\alpha$  accounts for the molecular weight differences between GSK3 $\alpha$  (51 kDa) and GSK3 $\beta$  (47 kDa) (Woodgett, 1990). The last 76 amino acids within the C terminus region of GSK3 $\alpha$  and GSK3 $\beta$  exhibit only 36% homology (Woodgett, 1990). It is likely that these structural differences between GSK3 $\alpha$  and GSK3 $\beta$  contribute to their differential interactions

with signaling complexes, including the specific interaction of GSK3 $\alpha$ , but not GSK3 $\beta$ , with AKT. It is important to note that when GSK3 $\alpha$  was depleted in the supernatant after two cycles of immunoprecipitation, there was still a substantial amount of AKT in the supernatant, implying additional AKT complexes in the cells. It is possible that GSK3 $\alpha$ -AKT might be a specific complex preserved to regulate inflammatory response.

Tandem MS showed that GSK3 $\alpha$  phosphorylates AKT at T312, which is in the substrate binding site according to the structure of AKT kinase domain. The phosphorylation on the side chain hydroxyl group of T312 will cause steric clash with the substrate, thereby inhibiting the AKT kinase activity. Interestingly, whereas GSK3 $\alpha$  deficiency enhanced IL-1-induced phosphorylation of the activation loop at T308, GSK3 $\alpha$  kinase-active knockin blocked IL-1-induced phosphorylation at T308. Although the phosphorylation of T308 is essential for AKT activation, the activation loop does not overlap with the substrate binding site. Therefore, it is not clear how the phosphorylation at T312 affects the phosphorylation of the activation loop at T308. A possibility is that the interaction of GSK3 $\alpha$  with AKT and/or phosphorylation at T312 might interfere with the access of AKT to the kinase (e.g., PDK1 and/or IKKi, discussed below) for the phosphorylation of the activation loop of AKT.

Consistent with the fact that PI3K and AKT inhibitors failed to inhibit IL-1-induced GSK3 $\alpha$  phosphorylation (S21), IKKi deficiency impaired IL-1-induced GSK3 $\alpha$  phosphorylation (S21), implying the possibility of IKKi as the upstream kinase for GSK3 $\alpha$ . In support of this, *in vitro* kinase assays clearly indicate that IKKi indeed can directly phosphorylate GSK3 $\alpha$ . Interestingly, tandem MS showed that IKKi phosphorylates GSK3 $\alpha$  at S21, suggesting that IKKi is responsible for IL-1-induced GSK3 $\alpha$  phosphorylation at S21. GSK3 $\alpha$  S21 is a known inhibitory phosphorylation site for GSK3 $\alpha$  kinase activity, which mainly blocks the binding of GSK3 $\alpha$  to its "primed substrates" (Frame et al., 2001). Most of the best-described GSK3 substrates require prephosphorylation at a residue 4 or 5 amino acids (or further) C-terminal to GSK3 target residue, a phenomenon referred to as "priming" (Fiol et al., 1987). Although it is unclear whether AKT is a primed substrate, GSK3 $\alpha$  S21 to A21 attenuated IL-1-induced AKT phosphorylation, presumably as a result of prolonged kinase activity of GSK3 $\alpha$  S21A and consequent inhibitory phosphorylation of AKT at T312. It is interesting to note that AKT contains several glutamic acid residues (2, 7, and 10 amino acids) and aspartic acid residues (11 and 13 amino acids) C-terminal to T312, which might serve as priming residues for the phosphorylation of T312 by GSK3 $\alpha$ . Previous studies have shown that phosphorylation of S21 of GSK3 $\alpha$  results in the N-terminal domain of GSK3 interacting with its phosphate binding pocket, preventing recognition of primed substrates. We speculate that IKKi-mediated GSK3 $\alpha$  phosphorylation at S21 might inhibit the recognition of acidic amino acids C-terminal to T312, thereby blocking AKT T312 phosphorylation by GSK3 $\alpha$ . Future studies are required to test this hypothesis.

IKKi can directly mediate AKT phosphorylation and activation in response to EGF stimulation (Xie et al., 2011; Guo et al., 2011). In addition to IKKi's role in removal of the inhibition of GSK3 $\alpha$  on AKT, an important question is whether IKKi is the direct kinase for AKT phosphorylation and activation in response to IL-1 stimulation. We found that recombinant IKKi was able to effectively

phosphorylate AKT *in vitro*, indicating that the IKKi probably is able to directly phosphorylate AKT. However, because PI3K signaling is required for IL-1-induced AKT-mTOR activation, it is possible that PDK1 might also be involved in IL-1-induced AKT phosphorylation and activation. It is well known that PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3). Whereas AKT and PDK1 bind to PIP3 at the plasma membrane, PDK1 phosphorylates the activation loop of AKT at T308 (Alessi et al., 1997). Interestingly, our results showed that IL-1-induced phosphorylation of AKT T308 and downstream components of mTOR (p70S6K, S6, and 4EBP1) were abolished in the absence of PDK1. These results suggest that whereas IKKi is necessary for the phosphorylation of GSK3 $\alpha$  at S21 to remove the inhibitory effect of GSK3 $\alpha$  on AKT (T312), PDK1 might be the direct kinase responsible for AKT phosphorylation at T308. It is important to point out that PDK1 deficiency did not have much impact on IL-1-induced AKT phosphorylation at S473. Therefore, after the removal of the inhibitory effect of GSK3 $\alpha$  on AKT (T312), IKKi might directly phosphorylate AKT at S473 (Guo et al., 2011; Xie et al., 2011).

It is intriguing to note that PI3K and AKT inhibitors did partially inhibit IL-1-induced GSK3 $\beta$  phosphorylation at S9, which demonstrates the importance of PI3K-AKT in GSK3 $\beta$  phosphorylation. It is plausible that the activated AKT through the action of IKKi-GSK3 $\alpha$  in turn mediates the phosphorylation and inactivation of GSK3 $\beta$  (S9). Consistently, GSK3 $\beta$  was not detected in the IKKi complex and IKKi can only weakly phosphorylate GSK3 $\beta$  *in vitro*. As a matter of fact, previous studies have shown that because GSK3 $\beta$  inhibits mTOR activation through phosphorylation of TSC2 (Inoki et al., 2006), AKT-mediated GSK3 $\beta$  phosphorylation at S9 should alleviate the inhibitory effect of GSK3 $\beta$  on mTOR, contributing to mTOR activation. It is important to note that although IKKi is unable to phosphorylate GSK3 $\beta$  *in vitro*, IKKi deficiency abolished IL-1-induced GSK3 $\beta$  phosphorylation in Th17 cells. It remains unclear whether there is another kinase that mediates IL-1-induced GSK3 $\beta$  phosphorylation, because AKT inhibitor only partially blocked IL-1-induced GSK3 $\beta$  phosphorylation. Future studies are required to identify the candidate kinase(s) that participate(s) in IL-1-induced GSK3 $\beta$  phosphorylation.

Although it is well established that AKT phosphorylates and inactivates GSK3 $\alpha$  and GSK3 $\beta$  (e.g., in insulin signaling), we showed that GSK3 $\alpha$  can reversely phosphorylate AKT and suppress AKT activation in resting cells and that IL-1 stimulation activates IKKi to phosphorylate GSK3 $\alpha$ , resulting in inactivation of GSK3 $\alpha$  to promote AKT-mTOR activation. This study has been mainly carried out in Th17 cells, demonstrating the critical role of the IKKi-GSK3 $\alpha$ -AKT-mTOR axis in IL-1-dependent Th17 cell maintenance and/or proliferation and function. The kinase mTOR has emerged as an important regulator of the differentiation of helper T cells (Delgoffe et al., 2009, 2011). The mTOR complex promotes phosphorylation of the translational regulators S6K1 and 4E-BP1 and is believed to have a central role in regulating cellular growth and proliferation by modulating metabolism. In addition to the impact of mTOR-mediated 4E-BP1 on the translation of cell cycle regulators cMyc and Cyclin D1, recent studies indicate that mTOR activation is required for HIF1 $\alpha$  induction, a transcription factor driving the

glycolytic pathway (Shi et al., 2011; Dang et al., 2011). Whereas aerobic glycolysis has been associated with lymphocyte proliferation, HIF1 $\alpha$ -dependent glycolytic pathway plays a critical role in Th17 cell differentiation. We found that IL-1 stimulation also strongly induced HIF1 $\alpha$  expression in differentiated Th17 cells, which is specially blocked by rapamycin (data not shown). Future studies are required to elucidate how IL-1-induced mTOR activation leads to Th17 cell proliferation and effector function.

## EXPERIMENTAL PROCEDURES

### Mice

Wild-type mice were purchased from Taconic Laboratories. *Il1r*<sup>-/-</sup> mice were purchased from Jackson Laboratories. *Gsk3a*<sup>-/-</sup> mice, *Gsk3a*<sup>fl/fl</sup> mice, and *Gsk3a*<sup>21A/21A</sup> mice were generated as described (MacAulay et al., 2007; Doble et al., 2007; McManus et al., 2005). Mice were housed in the animal facility (SPF condition) at the Cleveland Clinic Foundation in compliance with the guidelines set by Institutional Animal Care and Use Committee.

### T Cell Differentiation

Naive CD4<sup>+</sup>CD44<sup>lo</sup> T cells from wild-type C57BL/6, CD4Cre*Gsk3a*<sup>fl/fl</sup>, and *Gsk3a*<sup>21A/21A</sup> mice were sorted by flow cytometry and activated with plate-bound 3 mg/ml anti-CD3 and 3 mg/ml anti-CD28 and in the presence of 5 ng/ml TGF- $\beta$  (Peprotech), 10 ng/ml IL-6 (Peprotech), 5 mg/ml anti-IL-4 (11B11), 5 mg/ml anti-IFN- $\gamma$  (XMG 1.2), 10 ng/ml IL-1 $\beta$  (R&D System), or combination of these stimuli. For intracellular staining, cells were stimulated with PMA and ionomycin in the presence of Golgi-stop for 4 hr, after which IL-17- and IFN- $\gamma$ -producing cells were analyzed with intracellular staining.

### Proliferation

Th17 cells were cultured (20  $\times$  10<sup>3</sup> cells/well) at a final volume of 200  $\mu$ l/well in 96-well plates. In some wells, rapamycin (50 nM) (Sigma) was added. After incubation for 96 hr, wells were pulsed with [methyl-3H]thymidine (1.0  $\mu$ Ci/well, specific activity 6.7 Ci/mM; New England Nuclear) and harvested 16 hr later by aspiration onto glass fiber filters. For proliferation assay, levels of incorporated radioactivity were determined by scintillation spectrometry.

### Quantitative Real-Time RT-PCR

Expression of the genes encoding IL-17A, IL-23R, IL-21, and ROR $\gamma$ t were quantified with the SYBER Green PCR Master Mix kit (Applied Biosystems) with primer pairs selected for amplification of each individual cytokine (Gulen et al., 2010). Relative gene expression was determined as the ratio of cytokine to  $\beta$ -actin gene expression levels for each sample.

### EAE Induction

Mice were immunized with MOG<sub>35-55</sub> plus complete Freund's adjuvant in conditions that induce active EAE. Th17 cells from lymph nodes of the immunized mice were restimulated with MOG<sub>35-55</sub> (20 mg/ml) and IL-23 (20 ng/ml) as described before (Gulen et al., 2010). After 4 days of culture, cells were collected and injected into recipient WT mice that had been sublethally irradiated (600 rads) at 4 hr before injection. Although 2  $\times$  10<sup>7</sup> total WT cells were transferred to each mouse, the numbers of *Gsk3a*<sup>-/-</sup> and *Ikke*<sup>-/-</sup> cells transferred were normalized against WT based on the ELISPOT analysis of the MOG-specific Th17 cell numbers after the cell culture (Figure S4). For the detection of IL-1 $\beta$  effect on the MOG-specific Th17 cells, cells were injected intravenously into irradiated (600 rads) *Il1r*<sup>-/-</sup> mice. The mice were intraperitoneally injected daily either with PBS or 400 ng IL-1 $\beta$  (R&D) for 7 days. After 7 days, ELISPOT analysis was performed for the MOG-specific IL-17-producing cells from spleens of *Il1r*<sup>-/-</sup> mice with mouse IL-17 ELISPOT kit (R&D) according to manufacturer instructions.

### Transfection, Coimmunoprecipitations, and Immunoblots

Procedures for transfection, coimmunoprecipitations, and immunoblots with 293 cells were previously described (Yao et al., 2007). Antibodies used in this study include primary anti-AKT, GSK3 $\alpha/\beta$  (Santa Cruz), GSK3 $\alpha$ , GSK3 $\beta$ ,

p-GSK3 $\alpha/\beta$ , p-AKT, p-4EBP1, 4EBP1, p-70S6K, p-I $\kappa$ B $\alpha$ , p-JNK, p-mTOR, mTOR, p-S6, p-TSC2, TSC2, PI3K, IKKi (Cell Signaling), and GAPDH (Ambion).

### In Vitro Kinase Assay

Recombinant proteins (GST-GSK3 $\alpha$  [CellSciences], GST-GSK3 $\beta$  [R&D], and GST-IKKi/IKKE [Active Motif]) or immunoprecipitated beads were subjected to in vitro kinase reaction as previously described (Bulek et al., 2011).

### Trypsin Digestion, LC/ESI-MS/MS, and MS Data Analysis

The detailed methods for Trypsin digestion and LC/ESI-MS/MS have been previously described (Pang et al., 2002; Gao et al., 2004). MS/MS data were searched against human database by Sequest through Sorcerer (Sage-N Research, Milpitas, CA). The searched data set was processed by TPP (Trans-Proteomics Pipeline) and filtered with Peptide Prophet (Keller et al., 2002) as well as Sorcerer Sequest scores (Xcorr > 2.0, DelCN > 0.095, Rsp < 5) (Pang et al., 2002; Gao et al., 2004). Additionally, the phosphopeptides that passed the filtering criteria were manually inspected to confirm the assignments generated by the data searching algorithm.

### Statistics

ANOVA was used for EAE clinical score studies. The Student's t test was used to assess all other statistical values. p values were determined and error bars represent standard error of the mean (SEM).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2012.08.019>.

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